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Protein-loaded poly(DL-lactide-co-glycolide) microparticles for oral administration: formulation, structural and release characteristics

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Abstract

FITC-labelled bovine serum albumin has been entrapped in sub-5 micron particles of poly(DL-lactide-co-glycolide copolymer) (PLG) using a water-in-oil-in-water (w/o/w) emulsification-solvent evaporation technique. The concentration of PVA stabiliser in the external continuous phase was found to affect not only the particle size, size distribution and protein content but also the release characteristics and internal structure of the microparticles. The importance of primary emulsification was underlined by the finding that the protein content of microparticles with mean size 1 μm could be increased from about 1% w/w to around 12% w/w by increasing the amount of protein added to the primary emulsion and the homogenisation time in this stage. Under conditions of low stabiliser concentration, multi-nucleate particles formed by polymer precipitation and envelopment of the droplets of the primary w/o emulsion. In this case surface protein loading was of the order of 30% w/w. Under conditions of high PVA stabiliser concentration, disruption of the primary emulsion occurred, resulting in sub-micron particles which were characterised by a high surface protein loading of the order of 70% w/w. A mechanism for protein microencapsulation is presented which is heavily influenced by the shear stresses induced during the process of secondary emulsification. This can explain certain aspects of the relationship between microparticle size and size distribution, protein content and release and the structural characteristics of microparticles produced using the w/o/w emulsification/solvent evaporation technique.

Keywords: Poly(lactide co-glycolide); Protein-loaded microparticles; Oral delivery

1. Introduction

Microencapsulation of therapeutic polypeptides such as luteinising hormone releasing hormone (LHRH) agonist and calcitonin offers the possibility of controlling the pattern of release and pharmacokinetics of drug absorption [1,2]. A large number of natural and synthetic polymers are potentially suitable for production of the wall-forming polymer but the synthetic biodegradable poly(lactide-co-glycolide) copolymers (PLG) are among the primary candidates because of excellent tissue biocompatibilty, biodegradability and regulatory approval [3].

Microencapsulation has also been widely investigated for delivery of vaccines, eliciting impressive immune responses after both sub-cutaneous and oral delivery [4,5]. In the case of sub-cutaneous immunisation, the adjuvanticity of microencapsulated antigen is considered to result from efficient phago-

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cytosis of sub-10 μm microparticles, transport to the lymph nodes and gradual release of antigen to stimulate immunocompetent cells [6]. Sub-cutaneous or intra-muscular administration of microparticles is less demanding than oral delivery in terms of the size of the delivery vehicle with nanoparticles and 10 μm particles being suitable for injection.

Observations of particle uptake by the lymphoid aggregate tissue or Peyer's Patches (PP) in the gastrointestinal (GI) tract, suggest that oral administration of microencapsulated vaccines is feasible [7]. Following uptake by the PP epithelium-antigen sampling cells (known as M cells), microencapsulated antigen would be transported from the intestinal lumen to the underlying follicle, where B-cell and T-cell responses are induced [8]. Both mucosal and systemic immune response have been reported after oral administration of microencapsulated antigen to mice [8–10]. Protection of antigens against the acidic and enzymatic conditions of the GI tract and presentation of the antigen in particulate form to M cells are considered the advantages of microencapsulating antigen for oral delivery.

Although the importance of size on particle uptake is well appreciated, the optimum particle size range for uptake by the PP is still a matter for conjecture. However, it is apparent that particles of more than 10 μm in size cannot be taken up. In early reports, Eldridge et al. [7] showed that polystyrene particles below 5 μm diameter were found in the mesenteric lymph nodes while particles above 5 μm diameter were adsorbed on the mucosal and submucosal layer of PP in mice. Jani et al., [11] reported that 1 μm diameter polystyrene (PS) particles were taken up to a lesser extent than smaller particles in the rat and therefore considered that 1 μm should be regarded as the largest size of microparticles for oral delivery of protein drugs or antigens. Polystyrene particles of 3 μm diameter were not detected in the internal structure of PP but were found to be adsorbed to the submucosal layer of PP. The results of Jenkins et al. [12] showed greater numbers of 0.5 μm and 3 μm PS particles in the PP of the rat compared with smaller (0.15 μm) and larger (10 μm) particles which is in agreement with the findings of Eldridge et al. [7]. Thus, the use of antigen-containing particles, less than 5 μm in diameter, is more likely to lead to a better particle uptake by the PP which could improve the prospects for a beneficial therapeutic response.

A process of emulsification followed by solvent evaporation/extraction is the most widely used technique for preparing microparticles containing water soluble drugs and proteins [13–15]. The use of a simple oil in water (o/w) technique to encapsulate water soluble drugs, proteins and peptides usually results in large particles and non-uniform protein release. Poor encapsulation efficiency also remains a major problem due to a very low solubility of polypeptides in the organic phase [13,14]. Ogawa et al. [15] developed a water-in-oil-in-water (w/o/w) emulsion technique, based on the method of Vranken and Claeys [16], to encapsulate proteins and peptides with higher efficiency. A primary (w/o) emulsion was formed by mixing an aqueous solution of the protein with the polymer solution. The primary emulsion was subsequently mixed with an aqueous surfactant phase to produce a double emulsion. Since then many publications have documented the effects of microencapsulation conditions on microparticle size and size distribution, protein loading and protein release rate [17–21]. However, some fundamental aspects of microencapsulation remain unclear such as the distribution of core- and surface-located protein which may exert a profound effect on the immune response, particularly after oral administration. For example, degradation of exposed surface protein would reduce the effective delivered dose. Of more importance, however, is the potential in the GI tract for a SIgA response to surface protein which would be expected to negate the effect of any repeat oral administration of the microparticles.

The aim of the work reported here was to determine the formulation conditions suitable for production of protein-loaded PLG microparticles having a size and size distribution potentially suitable for oral delivery of antigens and particle uptake across the GI tract (i.e. <5 μm). The effect of processing conditions on the level of entrapment, location and release rate of a model protein antigen, BSA, was also determined to provide design and formulation guidelines for delivery systems for oral vaccines based on resorbable microparticles.

2. Materials and methods

2.1. Materials

The 75:25 poly (DL-lactide-co-glycolide) (75:25 PLG) (Mw 17 000, Resomer RG 755) was obtained from Boehringer Ingelheim. Poly(vinyl alcohol) (PVA, Mw 13–23 000, 80% hydrolysed) was obtained from Aldrich, bovine serum albumin (BSA) and fluorescein isothiocyanate bovine serum albumin (FITC-BSA) was supplied by Sigma. HPLC grade dicholoromethane (DCM) was purchased from Fisons and Metset resin and hardener type FT were purchased from Buehler.

2.2. Preparation of protein-loaded microparticles

A 1–2 ml mixture of BSA and FITC BSA (3:1) (20–40 mg/ml) was emulsified at high speed for 2–4 min using a Silverson homogeniser (12 400 revs./min) (Silverson Machines, Chesham, Bucks, UK) in 10 ml of a solution of PLG in DCM (1–6% w/v) to form a primary w/o emulsion. Then, 20 ml of an external water phase containing 1–10% w/v PVA as a particle stabiliser was added and homogenisation was continued for a further 4 min. The resulting suspension was stirred with a magnetic stirrer under ambient conditions overnight to allow the organic solvent to evaporate and the microparticles to harden. The resulting microparticles were harvested and cleaned by centrifuging and washing with distilled water a total of three times. Finally the microparticles were freeze dried and stored in a dessicator below 5°C.

2.3. Protein content of microparticles

The protein content of microparticles was analyzed using a method similar to that reported by Hora et al. [21]. Briefly, digestion of microparticles was achieved by treating 7–10 mg of lyophilised microparticles with 3 ml of a 5% SDS in 0.1 M NaOH solution for at least 16 h at room temperature until a clear solution was obtained. The protein content of at least three samples per batch was measured spectrophotometrically at 488 nm and confirmed using a BCA protein assay.

2.4. Protein release studies

First, 15–20 mg of dried microparticles were accurately weighed in culture tubes and resuspended in 3 ml phosphate buffered saline (PBS, pH 7.4) containing 0.02% sodium azide as a bacteriostatic agent. The microparticle suspensions were stirred continuously at 37°C in a water bath using an immersible magnetic stirrer. After 30 min and then at intervals of 24 to 72 h the samples were centrifuged, the supernatant was removed and analyzed for protein content using a spectrophotometer at 488 nm and confirmed using a BCA protein assay. Fresh release medium was added to the microparticles and the release studies were continued over a period of 4 weeks.

2.5. Analysis of surface protein

The analysis of surface protein was based on the displacement of surface-adsorbed protein by sodium dodecyl sulfate (SDS) — a powerful negatively charged detergent — that binds to the hydrophobic regions of protein molecules [22] causing them to unfold into extended polypeptide chains and freeing them from their associations with other molecules. First, 5–7 mg of microparticles were accurately weighed and resuspended in 2 ml of 2% (w/v) SDS solution and agitated for 4 h at room temperature using a IKA Vibrax VXR (IKA-Labortechnik, Staufen, Germany). The samples were centrifuged and the supernatant was analyzed for protein. At least three samples of microparticles were assayed for each formulation.

2.6. Light and fluorescence microscopy

Confocal laser scanning microscopy (CLSM) was used to investigate the structure and morphology of protein-loaded PLG microparticles. Microparticles were immobilised on glass slides using a 10% w/v gelatin solution and examined by Confocal microscopy using a BioRad-MRC 600 (BioRad Microscience Division, Hemel Hempstead, UK), a ×100 objective (NA 1.25, oil immersion), 488 nm excita-

tion with the BHS filter block and a transmitted light detector.

2.7. Particle sizing and scanning electron microscopy

The particle size and size distribution was recorded using a Malvern Mastersizer 2600 D (Malvern Instruments, Malvern, UK) for microparticles above 0.7 μm. The mean particle size in this case represents the volume mean diameter of the microparticles, and was obtained from measurements of at least three batches of microparticles unless stated otherwise. The size of microparticles less than 0.7 μm was measured by Photon Correlation Spectroscopy (PCS) (Malvern Instruments). Each sample was analysed at least six times to give an average value and standard deviation for the particle diameter.

The surface and internal structure of microparticles were examined by scanning electron microscopy (SEM; Jeol 6400, Tokyo, Japan). Samples were prepared by dropping a microparticle suspension in distilled water onto aluminium stubs and allowing them to air dry before coating with gold. Sectioning of particles was carried out after dispersing the microparticles in Metset resin blocks. Thin sections of the resin were prepared using an ultramicrotome and mounted on aluminium SEM stubs.

3. Results and discussion

3.1. Influence of process parameters on protein entrapment and microparticle size

3.1.1. PLG copolymer and PVA stabiliser concentration

BSA loaded microparticles were prepared using various concentrations of PLG solution (1–6% w/v) and PVA stabiliser solution (1–10% w/v) to investigate their effect on particle size, protein loading and efficiency of entrapment. The results are presented in Table 1.

In general, the protein entrapment efficiency was improved by increasing PLG copolymer concentration from 1 to 6% at a constant PVA stabiliser concentration. This is in agreement with the findings of Ogawa et al. [15] and Yan et al. [20] and is considered to be related to the high concentration of polymer in the emulsion droplets which tends to restrict migration of the inner aqueous/protein phase to the external water phase. In line with the empirical relationship between particle size and stabiliser concentration/viscosity, [14], a gradual increase in microparticle size from 0.38 to 1.13 μm occured in the 3% PLG system as the concentration of PVA in the continuous phase was reduced from 10 to 1% w/v. This trend was accompanied by a gradual and substantial improvement of protein loading and

Table 1
Effect of PLG and PVA stabiliser concentration on particle size, protein loading and entrapment efficiency

PLG conc. (% w/v)	PVA conc. (% w/v)	Mean size (μm)	Protein loading (% w/w)	Entrapment efficiency (% w/w)
6	10.0	0.58 ± 0.12	3.0 ± 0.2	78.2 ± 5.2
	5.0	1.08 ± 0.03	3.2 ± 0.1	95.6 ± 3.0
	2.5	1.27 ± 0.09	2.5 ± 0.1	75.2 ± 3.0
	1.0	1.72 ± 0.11	3.3 ± 0.4	85.6 ± 9.0
3	10.0	0.38 ± 0.04	1.4 ± 0.1	19.1 ± 1.6
	7.5	0.55 ± 0.03	3.2 ± 0.6	37.5 ± 7.0
	5.0	0.76 ± 0.17	3.8 ± 0.5	59.2 ± 7.8
	2.5	1.02 ± 0.21	4.8 ± 0.4	74.3 ± 6.2
	1.0	1.13 ± 0.06	4.9 ± 0.5	66.3 ± 6.8
1	2.5	0.71 ± 0.01	1.7 ± 0.1	6.5 ± 0.4

Homogenisation speed 12 400 revs./min.

entrapment efficiency by a factor of 3. At higher polymer concentration (6% PLG), changes in PVA concentration had less effect on protein loading characteristics. The protein entrapment efficiencies were consistently high (>75%) and protein entrapment only varied from 2.5% to 3.3% w/w. As in the 3% PLG system, the microparticle size increased with a reduction in stabiliser concentration. The same trends were reported by Jeffery et al. [17] for ovalbumin (OVA)-loaded PLG microparticles.

In the case of microparticle production using 3% PLG copolymer solutions, reducing the homogenisation rate during secondary emulsification, resulted in a substantial (×3) improvement in protein loading and efficiency when high (10%) PVA stabiliser concentrations were used in the process (Table 2). Changes in protein loading and entrapment efficiency were less marked on reduction of the homogenisation rate when a low PVA stabiliser concentration (2.5%) was used (Table 2). These effects demonstrate a relationship between PVA viscosity and homogenisation rate which combine to influence the shear stress experienced by the emulsion and consequently the protein association with the microparticles.

Improvements in protein loading and entrapment efficiency of PLG microparticles were obtained by increasing the time of primary emulsification from 2 to 4 min (Table 3). These improvement were more significant at lower concentrations of PLG copolymer. The creation of a fine dispersion of protein-containing polymer droplets in the primary emulsion is indicated and an overall improvement in primary emulsion stability, which, as suggested by Nihant et al. [23] is expected to enhance the loading of hydrophilic drugs.

BSA loading was also found to be affected by the concentration of protein solution in the internal aqueous phase. A general increase in protein loading of the microparticles is evident as the amount of protein in the internal aqueous phase is increased (Table 3), but entrapment efficiency can decrease due to a higher percentage of unencapsulated protein. In comparison, Prieto et al. [19], reported an increase in peptide loading and a slight decrease in encapsulation efficiency as the concentration of peptide in the internal aqueous phase increased. Jeffery et al. [17] reported an increase in OVA entrapment in PLG microparticles by at least a factor of 2 on doubling the initial weight of OVA in the internal aqueous phase.

3.1.2. Effect of PLG and PVA stabiliser concentrations on particle size distribution

When BSA-loaded microparticles were prepared from 3% PLG solutions, a decrease in the concentration of PVA was found to result in an increase in particle size and size distribution but a monomodal size distribution was maintained over the range of PVA stabiliser concentrations investigated (1–10% w/v) (Fig. 1). In contrast the use of a higher concentration of (6% w/v) PLG copolymer for production of BSA loaded microparticles, resulted in a bimodal size distribution (Fig. 2) as the PVA stabiliser concentration was reduced from 10 to 1%

Table 2
Effect of secondary emulsion homogenisation speed and PVA concentration on microparticle characteristics

PVA conc. (% w/v)	Homogenisation speed (revs./min)	Mean size (μm)	Protein loading (% w/w)	Entrapment efficiency (% w/w)
10	12 400	0.38 ± 0.04	1.4 ± 0.1	19.1 ± 1.6
	9800	0.43 ± 0.03	4.0 ± 0.1	48.0 ± 0.7
	7200	0.51 ± 0.02	4.2 ± 0.2	56.1 ± 2.3
2.5	12 400	1.02 ± 0.21	4.8 ± 0.4	74.3 ± 6.2
	9800	1.26 ± 0.09	5.4 ± 0.8	78.0 ± 2.8
	7200	1.43 ± 0.01	6.7 ± 1.1	69.2 ± 2.5

3% PLG concentration.
Primary emulsion homogenised at 12 400 revs./min.

Table 3
Effect of PLG concentration, primary emulsion homogenisation time and protein amount/volume on protein loading

PLG conc. (% w/v)		Homogenisation time (min)	Protein (mg) (volume, ml)	Mean size (μm)	Protein loading (% w/w)	Entrapment efficiency (% w/w)
1		2	20 (1)	0.7	1.7 ± 0.1	6.5 ± 0.4
	*	4	20 (1)	0.9	8.2 ± 0.1	34.9 ± 0.3
	*	2	40 (2)	0.7	9.5 ± 0.3	22.2 ± 1.2
	*	4	60 (1)	1.4	12.0 ± 0.9	19.5 ± 1.5
2	*	2	20 (1)	1.1	1.1 ± 0.1	5.7 ± 1.1
	*	4	20 (1)	1.1	6.3 ± 0.3	50.4 ± 2.2
	*	4	40 (2)	1.1	12.2 ± 0.2	60.6 ± 1.2
3		2	20 (1)	0.8	4.8 ± 0.4	74.3 ± 6.2
	*	4	20 (1)	1.0	6.0 ± 0.2	85.9 ± 3.1
	*	2	40 (1)	0.9	7.1 ± 0.4	52.6 ± 1.5
	*	4	40 (1)	1.0	7.6 ± 0.3	57.2 ± 1.3
	*	2	40 (2)	1.2	7.0 ± 0.1	51.3 ± 0.2
6	*	2	20 (1)	1.3	2.5 ± 0.1	75.2 ± 3.0
	*	4	20 (1)	1.2	2.7 ± 0.1	79.3 ± 1.5

*Measurments obtained from a single batch of microparticles at each set of formulation conditions.
Primary and secondary emulsion homogenised at 12 400 revs./min.
PVA concentration 2.5% w/v.

w/v. The results of Ogawa et al. [15] and Prieto et al. [19] also reveal a bimodal size distribution in w/o/w microparticulate formulations prepared using high concentration of PLG solutions in DCM (≥10%) and low concentrations of PVA (0.5% and 1.0% w/v respectively) in the external water phase. These effects probably arise from non-uniform droplet size reduction which may be influenced by a variable content of the aqueous protein phase in the polymer droplets, resulting in non-uniform shrinkage of the microparticles [24] and a heterogenous size distribution.

3.1.3. Microparticle morphology

The SEM micrograph of BSA-loaded microparticles (Fig. 3) reveals the wide particle size distribution characteristic of samples prepared using 6% PLG solutions and low concentration (1%) PVA solutions. (The particle size distribution in samples

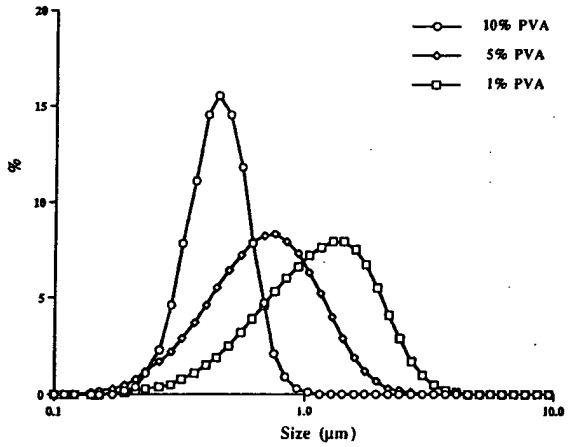

Fig. 1. Effect of stabiliser concentration on microparticle size distribution (3% PLG concentration).

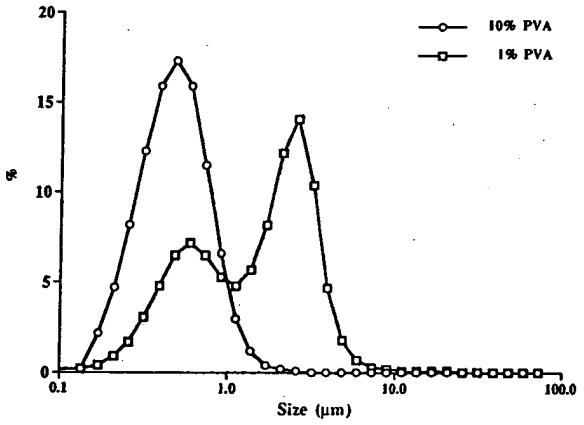

Fig. 2. Effect of stabiliser concentration on microparticle size distribution (6% PLG concentration).

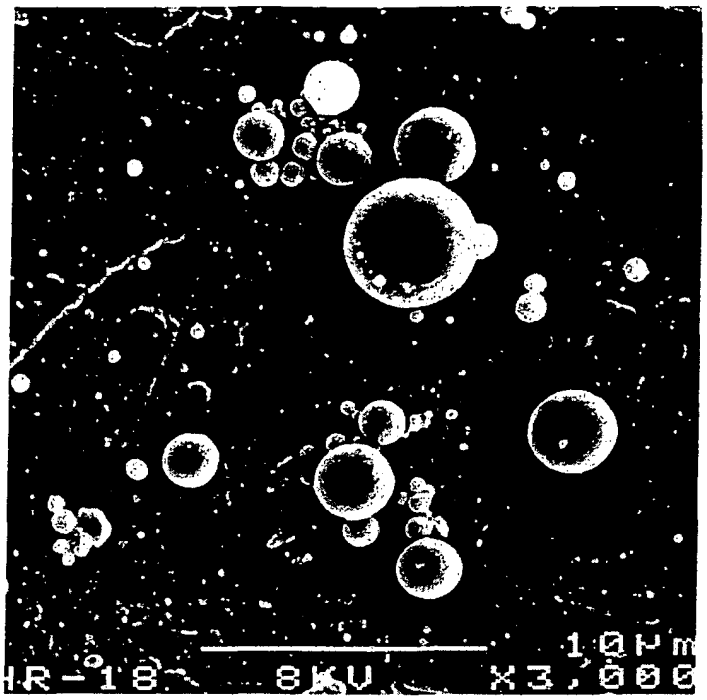

Fig. 3. SEM of BSA-loaded PLG microparticles prepared using 6% PLG and 1% PVA stabiliser solution.

prepared under these condition was bimodal corresponding to a population of microparticles around 1 μm and 2.4 μm, respectively (Fig. 2)). Samples prepared using high PVA concentrations (10%) are characterised by a more uniform particle size distribution (Fig. 4) and a mean particle size of less than 1 μm (see also Fig. 2).

Investigations of microparticle structure using CLSM revealed multinucleate particles containing discrete sub-micron protein islands (Fig. 5) when a low stabiliser concentration (1% PVA) and 6% PLG solution were used to prepare microparticles. The individual regions of protein within the microparticle appear roughly spherical and of the order of 0.1–0.5 μm. Fig. 6 shows a Z-section sequence through a FITC-BSA-loaded, multinucleate microparticle, at 0.1 μm steps. The 12 sections shown in Fig. 6 indicate a random dispersion of the protein zones throughout the microparticle.

The scanning electron micrograph of a sectioned multinucleate particle in Fig. 7 reveals the continuous surface envelope of the PLG microparticles. The internal structure appears to consist, however, of a high density of cavities and 'core particles', which may correspond to the primary emulsion (w/o) droplets, embedded in a polymer matrix. Protein could be bound to the surface of the cavities in the dehydrated form.

Similar microstructures and explanations of their origin have been reported by Yan et al. [20] and Prieto et al. [19], although the particle size in both cases was in excess of that considered satisfactory for oral vaccines. The macroparticle structure of 30 μm ricin toxoid-loaded PLG particles produced by Yan et al. [20] was considered to result from a cluster of hydrophilic ricin toxoid in smaller core particles (approximately 3 μm in size) encapsulated by a continuous polymer coat. Prieto et al. [19] produced peptide loaded PLG microparticles ranging from 4–17 μm in size that were found by electron microscopy, to contain several cavities (approximately 5 μm and below) separated by polymer matrix. Most of the cavities contained particles which were considered to be agglomerates of peptide molecules. In related work, Sah et al. [25] found that the microstructure of BSA-loaded PLG microparticles

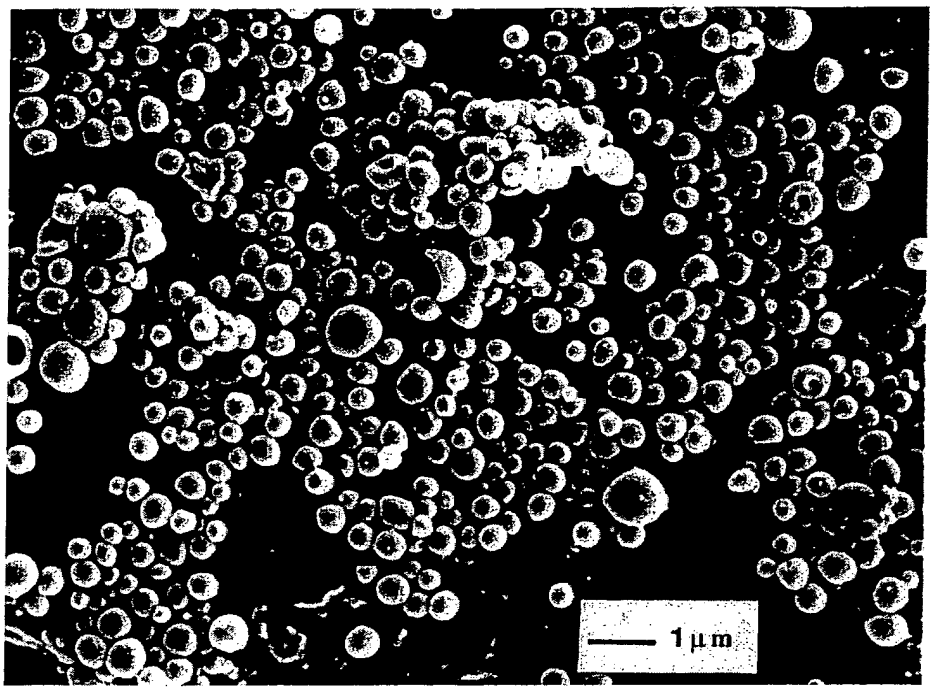

Fig. 4. SEM of BSA-loaded PLG microparticles prepared using 6% PLG and 10% PVA stabiliser solution.

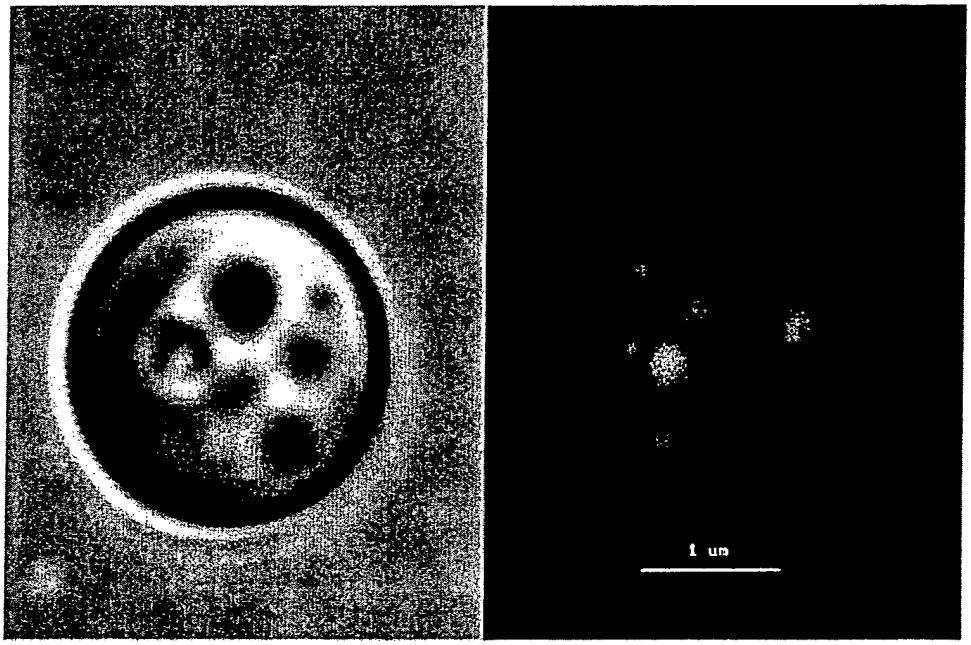

Fig. 5. Confocal view of a multinucleate microparticle prepared using 6% PLG, 1% PVA stabiliser solution and FITC-BSA. (Two images in register. Left transmitted light and right flourescence image).

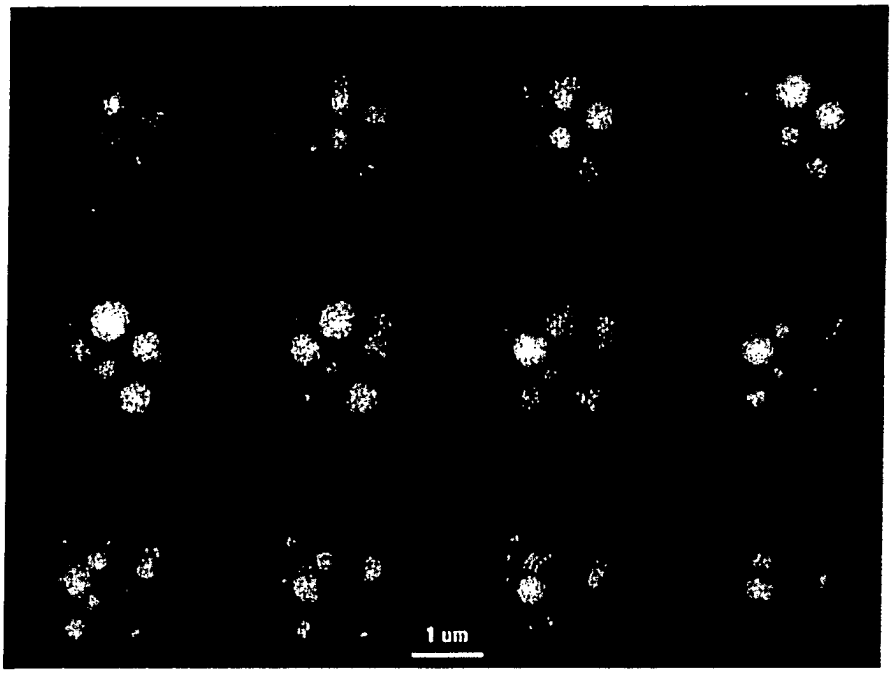

Fig. 6. CLSM view of internal structure of a single multinucleate microparticle showing distribution of FITC-BSA.

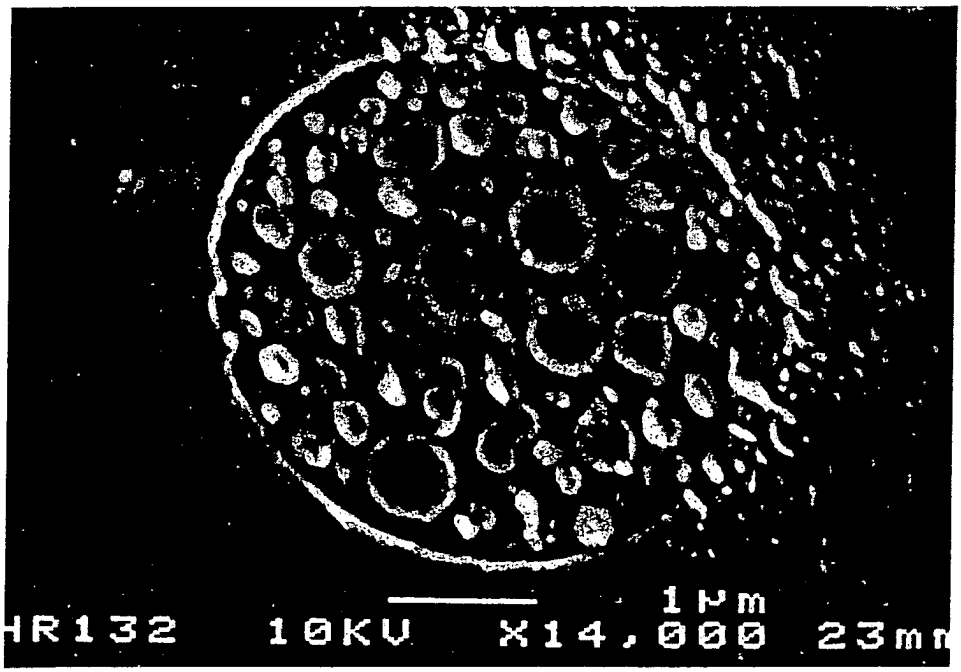

Fig. 7. SEM showing the internal structure of sectioned, multinucleate microparticles prepared using 6% PLG and 1% PVA stabiliser concentration.

could be influenced by the magnitude of the shear rate applied to the primary emulsion. High shear rates during the primary emulsification, produced large numbers of small cavities in 10–75 μm size range microparticles.

3.1.4. Protein release characteristics

The protein release profile of BSA-loaded microparticles prepared using 6% PLG solutions and 1 and 10% PVA, respectively, are shown in Fig. 8. Both types of microparticles exhibit a burst phase of protein release in the early stages of testing amounting to approximately 30% and 50% of the protein loading for the 1% PVA system and 10% PVA system, respectively. The burst release of protein is normally considered to be due to the surface-located protein [26]. It appears that the greater surface area of the smaller sub-micron particles or partial break down of the emulsion, under the influence of high shear stresses and the increased viscosity of the PVA stabiliser solution results in microparticles having more surface bound protein. The results of Yan et al. [20] obtained using BSA-loaded PLG microparticles prepared by a w/o/w technique may indicate similar effects arising from shear induced breakdown of emulsions. An initial 7.4% burst release of protein occured from (24 μm) microparticles when vortex mixing was used to prepare the secondary emulsion (94.3% entrapment efficiency). A higher amount (35.6%) of protein was released from smaller 7 μm microparticles when sonication was used for preparation of the secondary emulsion (60.1% entrapment efficiency).

Protein release from the microparticle core will depend upon various factors such as the degradation rate of the PLG copolymer matrix, denaturation and aggregation of protein molecules and polymer-protein binding. The release of core-loaded protein (if it occurs at all) is expected to be a long term process. Almost 80% of the BSA load was released from the sub-micron microparticles produced using 10% PVA stabiliser solution in 25 days (Fig. 8). In contrast a low rate of protein release occurred from microparticles prepared using 1% PVA, amounting to 40% of the protein loading. This behaviour can be related to the larger fraction of protein entrapped within the microparticle core.

Microparticles prepared using a 1% PVA stabiliser solution and having a bimodal size distribution (Fig. 2) were divided into two different groups of 1.01 μm (21.1 ± 0.4% w/w) and 2.36 μm (78.9 ± 0.3% w/w) particles by centrifugation at 2000 revs./min for 5 min. The resulting particles were washed and freeze dried as discussed above. The cumulative protein release from the fraction of small and large particles, respectively, is presented in Fig. 9. These protein release studies indicate that a major component of the protein load is located on the surface of

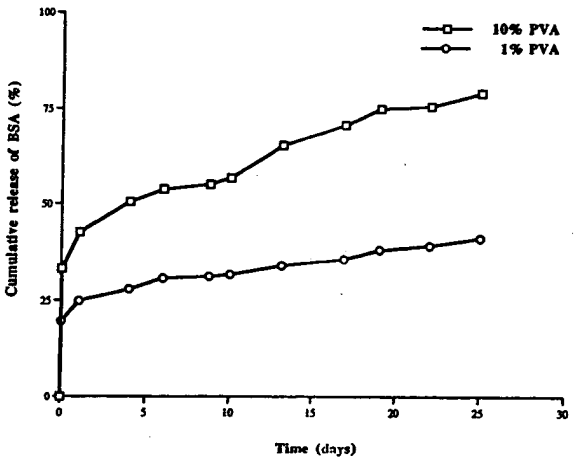

Fig. 8. Protein release from BSA-loaded microparticles prepared using 1% and 10% PVA stabiliser solution (6% PLG concentration).

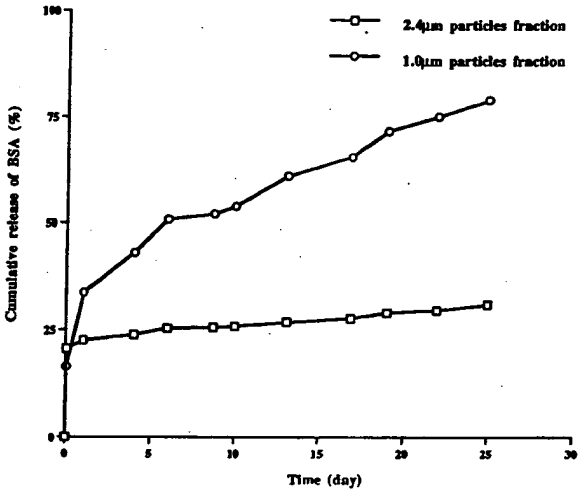

Fig. 9. BSA release from the microparticle fractions separated from a bimodal size distribution sample (6% PLG, 1% PVA).

the smaller 1.01 μm microparticles fraction (similar to sub-micron particles prepared using 10% PVA, Fig. 8) whereas a relatively low percentage of the protein load is removed from the surface of the 2.36 μm particle fraction.

The results of SDS surface protein analysis, presented in Table 4, also confirm that as the particle size is reduced to approximately 1 μm and below, for example by increasing the concentration of PVA in the external aqueous/stabiliser phase, less protein appears to be encapsulated. These findings are in line with those of Coombes et al. [27] who measured a surface ovalbumin (OVA) component on submicron OVA-loaded PLG microparticles equivalent to 50% of the total protein loading. It is well known that adsorbed proteins are difficult to remove from polymer surfaces even by detergent treatment [28]. Thus the level of surface protein detected by SDS treatment in the present study may be an underestimate of the actual surface protein component. Importantly, the above findings demonstrate that a population of small, sub-micron microparticles in a sample is likely to exhibit a large surface protein component which will influence the protein release characteristics and be susceptible to acid/enzyme degradation on oral administration unless suitably protected.

3.1.5. Encapsulation mechanism

The solvent evaporation/extraction technique consists of two main stages: formation of droplets of polymer solution by emulsification in an immiscible aqueous phase and solvent removal to cause microparticle hardening. During the first stage, droplets collide, coalesce and redivide continuously up to a steady-state point at which the droplet size remains stable. The use of a stabiliser solution provides a thin protective layer around the droplets and prevents coalescence and droplet destruction [29]. The gradual removal of solvent from the polymer solution droplets containing drug leads to polymer precipitation, and hence drug entrapment.

In the w/o/w system, an aqueous protein or peptide phase is first emulsified in the polymer solution prior to mixing with the external aqueous phase. Thus, in order to prevent the exposure of the internal aqueous/protein phase to the external aqueous stabiliser phase (leading to a reduction in protein entrapment), efficient encapsulation by the polymer is ideally required during primary emulsification (Stage 1, Fig. 10). Evidence for rapid stabilisation by polymer precipitation is provided by the findings of Bodmeier and McGinity [30] on microparticle production using an o/w emulsification/solvent evaporation technique. No further drug loss (suspended hydrophilic quinidine sulphate) occured from the organic phase, containing a high polymer concentration, to the aqueous stabiliser solution after the first minute of emulsification. Stabilisation of the primary emulsion containing aqueous/protein droplets in the w/o/w system may also be promoted by polymer precipitation during primary emulsification or surface interaction of polymer and protein as suggested by Nihant et al. [23].

Several recent publications have stressed the importance of the primary emulsification stage for increasing entrapment efficiency and for controlling the internal structure of microparticles prepared using the w/o/w emulsification solvent evaporation technique [19,20,25]. However the effect of the external aqueous/stabiliser phase on the dimensions

Table 4
Effect of manufacturing parameters on the location of protein in PLG microparticles prepared using the w/o/w technique

PLG conc. (% w/v)	PVA conc. (% w/v)	Mean size (μm)	Size distribution (10%< —90%<, μm)	Protein loading (% w/w)	Surface protein (% w/w)
6	10.0	0.58 ± 0.12		3.0 ± 0.2	67.1 ± 7.8
6	1.0	1.72 ± 0.11	0.4–3.3	3.3 ± 0.4	29.4 ± 3.9
	(a)	2.41 ± 0.05	1.7–3.8	3.9 ± 0.3	21.8 ± 1.6
	(b)	0.85 ± 0.22	0.7–1.6	1.4 ± 0.2	68.0 ± 3.2
3	2.5	1.02 ± 0.21	0.8–2.2	4.8 ± 0.4	43.0 ± 4.4

a, Large (multinucleate) particle fraction (79% w/w) separated by centrifugation at 2000 revs./min for 5 min.
b, Small particle fraction (21% w/w) remaining in supernatant after centrifugation at 2000 revs./min for 5 min.

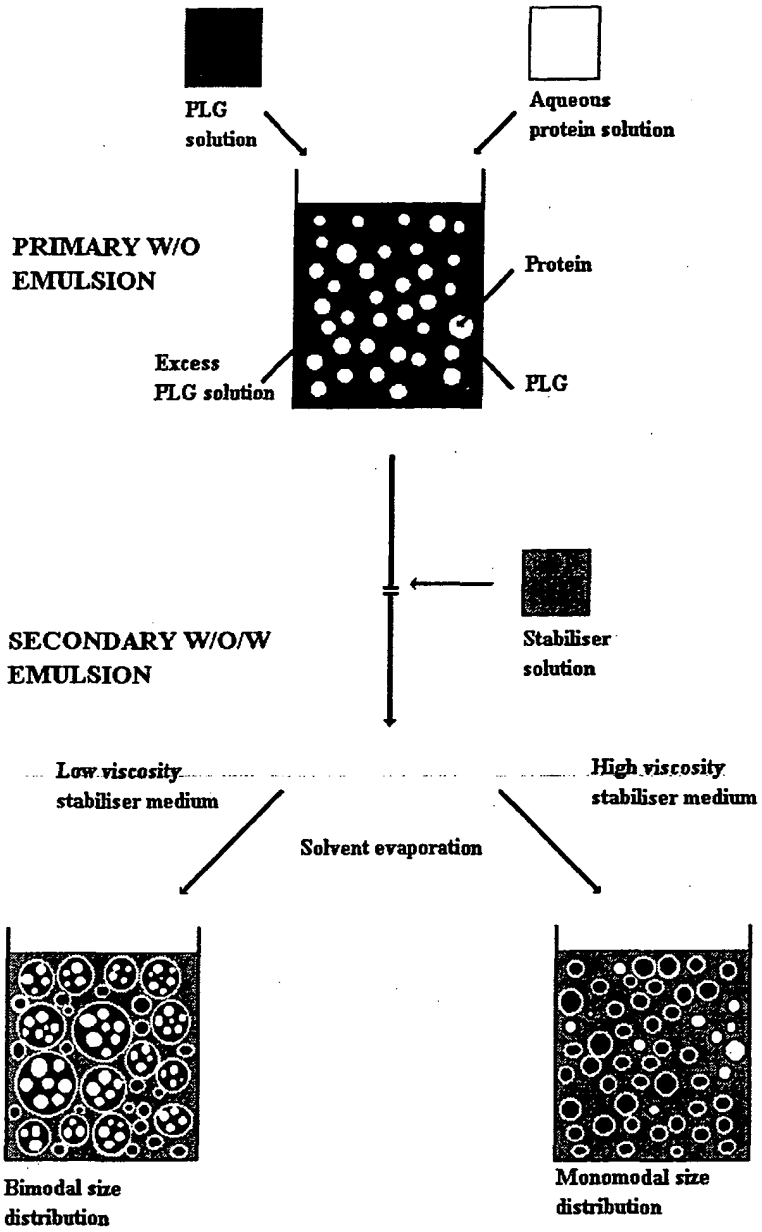

Fig. 10. Protein encapsulation in PLG microparticles under conditions of high and low stabiliser concentration.

of protein-loaded microparticles, their morphology and protein release characteristics has not been considered in detail.

Emulsification involves a complex interaction between applied mechanical forces (dependent on reactor design, homogenisation type, rate and uniformity) and the viscosity of the emulsion medium. These factors combine to affect the rheological behaviour of the emulsion and the shear stresses experienced by the droplets of polymer solution.The PVA surfactants exerts a dual effect on microparticle production. In addition to droplet stabilization, in-

creasing the concentration of PVA stabiliser in the external aqueous phase to 10% in this and other investigations [17,31] has been shown to result in a decrease in particle size by providing an efficient emulsification. Further increases in PVA concentration beyond a critical point (>15%) resulted in an increase in particle size in the w/o system [31] which was considered to arise from non-uniform homogenisation.

The proposed microencapsulation mechanism is summarised in Fig. 10. At low PVA concentrations in the external aqueous/stabiliser phase, large droplets of PLG solution containing small prestabilised droplets of protein solution are expected to be formed (Stage 2, Fig. 10). Under these conditions, the large droplets remain intact in the emulsion and further polymer precipitation may occur on the outer interface leading to multinucleate micoparticles or 'microcapsule aggregates' [20]. Fig. 5 and Fig. 6 and the internal structure of the multinucleate microparticles (Fig. 7) appear to confirm the mechanism of microparticle formation arising from use of low concentration surfactant solutions. In general, multinucleate microparticles tend to be formed by encapsulation of primary emulsion droplets under conditions of low shear rate and low stabiliser concentration. The present results demonstrate that this particular microencapsulation condition can be achieved in sub-5 micron microparticles.

However it is also apparent from the results presented here that the shear stresses generated in the double emulsion at high homogenisation rates and high PVA concentration, tend to divide the droplets of the primary emulsion and prevent encapsulation of the core particles formed during primary emulsification. The PVA surfactant molecules then induce stabilisation by forming a barrier around the droplets. Importantly, the latter formulation conditions result in reduced protein entrapment combined with the presence of a large fraction of protein on the surface of the particles (Table 4). The results of Yan et al. have also indicated the effect of high shear stresses (induced by sonication) during the secondary emulsification stage. Although the particle size measured by Yan et al. was reduced to around 6–7 μm, the entrapment efficiency was also reduced and the burst effect increased significantly to around 35%, indicative of a higher fraction of surface protein.

4. Summary and conclusions

The investigations reported here emphasise the requirement for careful control of formulation conditions, principally polymer solution concentration, stabiliser concentration and homogenisation rate in order to optimise core/surface protein ratio and thus to ensure minimal exposure of loaded protein. Sub-5 μm, BSA-loaded PLG microparticles were prepared using a w/o/w technique, exercising careful control over the secondary emulsification process. 'Multinucleate particles' containing discrete clusters of protein resulted when a low PVA stabiliser concentration was used. This structure is apparently formed by PLG envelopment of primary emulsion droplets. A surface protein loading of 30% (w/w) was measured. Shear induced disruption of the emulsion, under conditions of high PVA stabiliser concentration, promoted the formation of sub-micron particles. These were characterised by a major surface protein component of the order of 70%. The release characteristics of a microparticle sample have been found to reflect the change in population of sub-micron particles which exhibit predominantly surface protein loading.

The design and formulation of submicron microparticles for oral delivery would require further attention to achieve high core loading and thus protection of vaccine antigens and peptides from the acidic and enzymatic conditions existing in the GI tract. The elimination of surface protein is also desirable to eliminate SIgA responses to the delivery system which would reduce the prospects for take-up of repeat doses across the GI tract.

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